Effect of pH, sucrose and mannitol on structure and long-term stability of a model IgG1 antibody upon freeze-drying

By

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Chairperson Jennifer Laurence

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ABSTRACT:

Purpose: The purpose of this study is to investigate the effect of pH and potential stabilizers on structure and long-term stability of an IgG1 monoclonal antibody in the solid state after freeze-drying. The inter-relationships between preservation of secondary and tertiary structure of the protein in the solid state and long term storage stability under different formulation conditions and temperatures was obtained from examining data collected using spectroscopic techniques and stability-indicating assays, respectively.

Methods: Anti-Streptavidin IgG1 antibody was formulated with mannitol at pH 3.0, 5.0 and 7.0 in the presence and absence of sucrose as a stabilizer. All samples were stored at 4 ºC, 25 ºC, and 37 ºC up to 12 months and at 50 ºC for 6 months. Physical degradation of each lyophilized formulation was monitored using size-exclusion chromatography (SEC), covalent degradation was monitored using cation-exchange chromatography (CEX), and sub-visible particle counts (SbVP) were measured by HIAC. The secondary structure of the protein in the solid state was characterized using Fourier transform infrared (FTIR) spectroscopy and tertiary structure was monitored using fluorescence spectroscopy. Raman spectroscopy was also used to determine changes in secondary and tertiary structure spectral features.

Results: The IgG1 antibody underwent significant secondary structural perturbations at pH 3.0 regardless of excipients, and all formulations without sucrose also showed decreased structural stability in the solid state. Based on observation of structural changes, formulation at pH 5.0, showed the least change over time and at elevated temperatures. This correlated with long-term stability upon reconstitution with respect to protein aggregate formation and sub-visible particle counts.

Conclusions: The results of the study show that protein secondary and tertiary structural preservation in the solid state correlates to improved long-term stability of the monoclonal antibody in the different lyophilized formulations.

ACKNOWLEGMENTS

Thanks to Sampath Krishnan for being the Amgen advisor and Prof. Jenifer Laurence for being the advisor from University of Kansas, Prof. David Volkin for being part of the committee member, Vergara Camille for supporting with Raman data, Karthik Nagapudi for supporting with NMR data, Twinkle Christian, and Chris Woods for helping with FTIR analysis, Ranjini Ramanchander and Arnold McAuley for helpful discussions, and Dave Brems for the opportunity to be in this program.

TABLE OF CONTENTS

INTRODUCTION

Many therapeutic proteins are not stable in aqueous solution over long periods of time. Thus, freeze-drying is widely used for stabilizing therapeutic proteins and other biotechnology products for long-term storage.[1,2,3] Also, the industry is currently exploring freeze-drying as a particle mitigation strategy for certain therapeutic fusion proteins and monoclonal antibodies which otherwise form particles in the liquid state during long-term storage. Although stabilization techniques such as lyophilization are used to reduce the rate of chemical and physical degradation,[4] proteins can be unstable during lyophilization processes and/or during storage. Physical and covalent stabilization upon long-term storage is of fundamental concern because of the potential impact of degradation on immunogenicity, toxicity, and efficacy of the therapeutic protein drugs.[5] Therefore, different stabilizers such as sugars and polyols are normally added to the formulations to protect proteins against degradation during processing and storage,[6,7] but the processes by which sugars or polyols stabilize proteins in the solid-state are not completely understood.[1,2,8] Pikal et al.[9,10] and others[11,12,13,14,15] have investigated the mechanism of degradation and stabilization in the solid state, but more systematic evaluation of the effect of protein structure in the solid state on the long-term stability of proteins is needed as explained below.

The effect of structural changes and molecular mobility on long term stability of proteins in solid state formulations is implicated in two different proposed mechanisms by which excipients are believed to stabilize proteins.[5] The glass dynamic hypothesis states that a good stabilizer of freeze-dried formulations form a rigid, inert matrix into which the protein is molecularly dispersed and can couple the motion of protein to the motion of the matrix. α-relaxation of the glassy matrix occurs mainly due to translational motion, although rotational

motions are considered to strongly influence the diffusion of reactive molecular species within the matrix.[1,2,5] Therefore, protein unfolding and other degradation reactions that effect the physical stability of the protein would be expected to correlate with the molecular mobility within this matrix. Another proposed mechanism known as the water substitution hypothesis states that stabilizers such as sucrose or polyols hydrogen bond with the surface of proteins, thereby preserving the native structure when water is removed.[2,5,8] The removal of water in the absence of a sugar, often results in a non-native protein structure in the dry state, as has been detected using spectroscopic techniques, and may lead to aggregation during reconstitution.[4] In addition, it has been found that sugars provide a glassy matrix that reduces the rate of degradation during storage in the dried solid by preserving its native structure during lyophilization and storage. [3,4]

In the recent pharmaceutical literature, increasing evidence about the correlation between reactivity in the amorphous state and molecular dynamics[16,17] as well as structural changes has been proposed. Many studies have demonstrated that chemical degradation of amorphous pharmaceuticals is related to some degree of molecular mobility.[15,17] Therefore, reducing the molecular mobility of amorphous pharmaceuticals is considered to be a useful strategy for improving long term stability.[15] However, a reduction in molecular mobility does not always result in improved stability. There are various factors such as solute concentration, pH changes, and temperature that contribute to storage stability. Understanding the inter-relationships between chemical reactivity and molecular mobility of protein is critical predicting storage stability.[15] However, there are several examples in the literature where it has been shown that glass transition (Tg) and α enthalpic relaxation (time) are good predictors of lyophilized cake stability but not the long term storage stability of the protein. [14,15,17, 18] In such cases,

determination of fast dynamics, as measured by SANS & NMR techniques, has been shown to play a useful role in determining stability.[12,17,19,20]

Over the past 30 years, the maintenance secondary and tertiary structure of various proteins in the solid state has been investigated using a number of techniques including solid-state FTIR, Raman, NIR, fluorescence, phosphorescence spectroscopic techniques[21,22,23,24] as well as NMR. Secondary structure is important because formulations that retain more native-like structure are expected to have better storage stability than formulations having less native-like structure.[5] One particular advantage of Fourier transfer infrared (FTIR) spectroscopy is that this technique allows for secondary structure analysis of protein in a wide variety of physical states. FTIR is frequently used to assess structure of lyophilized proteins in stability studies and to guide the choice of the optimal formulation. During the evaluation of lyophilized protein formulations, the spectrum of each formulation can be compared with an aqueous reference spectrum with the goal of determining quantitatively the degree of retention of native secondary structure.[25] Raman spectroscopy can also be used to study protein conformation and to quantify the amount of protein secondary structure and, in some cases tertiary structure as well. [21,36,38]
Also, recent literature shows that solid-state fluorescence spectroscopy can be a useful for investigating the tertiary structure of proteins in solid powders and that tertiary structure correlates with long term stability.[26, 27]

The purpose of this study is to investigate the effect of pH and potential stabilizers such as sucrose and mannitol on structure and long-term stability of anti-streptavidin IgG1 monoclonal antibody in the solid state after freeze-drying. The relationship between preservation of the protein's secondary and tertiary structure in the solid state and the long-term storage stability was obtained from examining data collected using spectroscopic techniques and by

stability-indicating assays at different temperatures, respectively. The result of this study is expected to improve understanding of the role structural preservation has on long term stability of therapeutic proteins. This will enable development of tools to predict and generate stable lyophilized formulations.

MATERIALS AND METHODS

Materials and preparation of antibody formulations

Purified monoclonal antibody anti-SA IgG1 monomer (purity >97% determined by SEC, MW 142.2 kDa) was provided by Amgen, Inc. (Thousand Oaks, CA). The antibody was obtained at a stock concentration of 38.5mg/mL bulk in 10 mM sodium acetate and 5% sorbitol, at pH 5.0. The antibody was dialyzed into corresponding buffers and then diluted with different buffers to a final concentration of 5 mg/mL at different pH values, with mannitol in the presence or absence of sucrose. The buffer conditions used for various formulations are shown in Table 1. Sucrose was purchased from EMD (Gibbstown, NJ). Mannitol was purchased from Roquette (Keokuk, IA). Sodium citrate was obtained from JT Baker (Phillipsburg NJ). Each of the formulations at 1mL was filled in 3cc glass blowback vials (Amcor Type IA) with Daiko long stopper and lyophilized.

Table 1. Different formulation conditions along with measured pH and osmolarity values.

Sample Code	Buffer	Excipients	pH	pH (Experimental)	Osmolarity
C3M	10 mM sodium citrate	4% mannitol	3	3.07	295
C3MSu	10 mM sodium citrate	2 % sucrose, 4% mannitol	3	3.10	238
C5M	10 mM sodium citrate	4% mannitol	5	4.94	267
C5MSu	10 mM sodium citrate	2 % sucrose, 4% mannitol	5	4.99	250
C7M	10 mM sodium citrate	4% mannitol	7	6.74	251
C7MSu	10 mM sodium citrate	2 % sucrose, 4% mannitol	7	6.83	335

Lyophilization of Samples

The IgG1 proteins (5mg/mL) was formulated under each of the conditions described above. One-milliliter aliquots of each protein formulation were added to vials, which were lyophilized on a Virtis lyophilizer (SP Scientific, Stone Ridge, NY). Sample vials were loaded onto pre-chilled (4 ºC) shelves of the lyophilizer. Samples were held for 30 min at 4 ºC, cooled down to -45 ºC for 123 min, and held at -45 ºC for 180 min. Then, the shelf temperature was raised to -15 ºC over 150 min after holding at -15 ºC for 240 min. Finally, the temperature was lowered to -45 ºC again over 150 min after holding at -45 ºC for 120 min.

For primary drying, the shelf temperature was raised to -10 ºC and held for 25 hours while keeping the chamber vacuum at 120 mTorr after making the condenser temperature less than -50 ºC. For secondary drying, the shelf temperature was raised to 25 ºC over 234 min while lowering the chamber pressure to 100 mTorr. Then, the shelf temperature was maintained at 25 ºC for 11 hours at 100 mTorr. After all cycles were finished, the vials were stoppered inside the drying chamber. The vials were then removed and crimped before using them in long term stability studies.

Incubation study of formulations at different temperatures

Lyophilized samples were stored in incubators (Shel Lab, Cornelius, OR) equilibrated at different temperatures: 4 ºC, 25 ºC, 37 ºC, 50 ºC, -80 ºC (Liquid formulation for control). Different time points were set up for different analysis: 0 month, 1 months, 3 months, 6 months, 9 months, and 12 months. Three vials of each lyophilized formulation were pulled at each time point. Just prior to analysis, one vial from each condition was reconstituted with 1 mL of water.

pH , osmolarity and concentration measurements

pH was monitored using a MP200 Mettler Toledo pH meter (Columbus, OH). Samples were brought to room temperature before the analysis. The instrument was calibrated using pH 4.00 and pH 7.00 standard buffers. Osmolarity was determined using an Advanced Instruments, Inc (Norwood, MA) Micro Osmometer, model 330. 290 mOsM standards were used as a reference standard and water as a control. The actual pH and osmolarity values of the formulations are shown in Table 1.

Concentration was measured using ND-1000 spectrophotometer Nano Drop (Thermo Scientific, Wilmington, DE). A280 nm method was utilized with 1.06 (mL. $mg^{-1}.cm^{-1}$) extinction coefficient for the Anti- SA IgG1.

Karl Fisher analysis of moisture in lyophilized product

Residual moisture content of all lyophilized formulations was determined using Karl Fisher coulometric titration (Aquastar AQC34 KF Coulometer. (EMD, Gibbstown, NJ)). The lyophilized cakes were crushed into powder prior to analysis. 5% lactose was used as a standard.

Size-exclusion chromatography (SE-HPLC)

Size-exclusion chromatography was used as a stability-indicating assay with Agilent 1100 (Lexington, MA). The method employed one column (Tosoh G3000SWxl, 7.8 mm x 300 mm) attached with a guard column (TSKgel Guard Column SwXL 6.0 mm x 4.0 cm). Twenty micrograms of protein was loaded onto the column and eluted isocratically at a flow rate of 0.6 mL/min with a mobile phase consisting of 100 mM sodium phosphate, 300 mM sodium chloride; pH 6.8 at 25 ºC.

The protein was monitored using UV detection at 215 nm. Peak areas in the chromatogram were used to quantify the amounts of monomer, high molecular weight species (HMW), and low molecular weight species (LMW).

Visual inspection and SbV Particle Counting (HIAC)

HIAC particle counting was used to monitor aggregates in the 2-25 µm range, and large aggregates were qualitatively monitored by visual inspections. Liquid particle counting was performed on a HACH Ultra (Grants Pass, OR) HIAC model 9703 system using the PharmSpec software. Prior to analysis, samples were degassed under vacuum for one hour based on the method developed for monoclonal antibodies. It has been shown that degassing under mild vacuum helps release air bubbles that otherwise artificially increase the particle count. The degassing condition for 1 hour has not been found to cause physical damage for antibodies tested so far. Four 0.2 mL measurements were made for each sample and prior to sample

measurements Millipore water was used to blank the system. The first run was discarded, and the last three runs were averaged to obtain the cumulative counts per milliliter. As per USP guidelines, particle sizes of greater than 10 µm, and less than 25 µm were counted.

Cation-exchange chromatography (CEX)

CEX was carried at 25 ºC on a Dionex ProPac WCX-10 (4 x 250 mm) attached to a guard column (TSKgel Guard Column SwXL 6.0 mm x 4.0 cm) using an Agilent 1100 (Lexington, MA). Twenty micrograms of protein was loaded onto the column and eluted at the flow rate of 0.38 mL/min. Separation of various charged species was achieved through a gradient; mobile phase A was 10mM NaPO4, pH 7.1 and mobile phase B 10 mM NaPO4, 250 mM NaCl, pH 7.0. The mobile phase mixture varied from 3% at 8 min to 15% at 15 min, 17% at 20 min, 18.5% at 27.5 min, 21.5% at 42.5 min, 23% at 50 min, 40% at 53 min, 100% at 56 min to 58 min, and finally back to 0% from 60 min to 70 min. The protein was monitored using UV detection at 215 nm. The peak areas were integrated to quantify the amount of protein contained in the pre-peak (acidic variants), main peak and post peak (basic variants).

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectroscopy studies were conducted using Bomen TioTools MB-Series FTIR Spectrometer (ABB, Canada) to assess secondary structure in lyophilized solids.

Lyophilized powder was mixed with KBr to form a disc pellet. The solid samples were prepared by pressing a ground homogeneous mixture of 400 mg KBr with approx 4mg of dried protein formulation into a pellet using a mortar and pestle. This procedure has been shown to not cause any damage to proteins[25] let alone the more stable monoclonal antibodies. The mixture was pressed with EZ-press approximately 5500 psi and the pressure was maintained for

approximately 5 minutes. Then, spectra of samples in KBr were collected and analyzed using GRAMS software. A total of 64 scans was collected for each spectrum, and the scanning range was from 4000 cm^{-1} to 700 cm^{-1}. The second derivative of the amide I region (1720-1580 cm^{-1}) of the spectra was baseline corrected and normalized to an area of unity.

Solid-State Fluorescence.

All intrinsic tryptophan fluorescence measurements were made using a Cary-Eclipse spectrofluometer (Agilent, Australia). Studies were performed according to procedures described in the literature.[26] The lyophilized cake from each vial was broken and mixed together thoroughly with a spatula, and the sample was loaded into the solid-state holder. The solid-state holder (Cary-Eclipse) consists of a powder receptacle which houses the silica disk and shell cavity into which the lyophilized sample is placed. The silica disk in the shell cavity is completely covered with the sample to ensure reproducible measurement. A Photo multiplier tube (PMT) voltage of 600 V was used for the measurements involving lyophilized samples. The emission was monitored at 280-450 nm utilizing 280 nm wavelength and bandwidth of 5 nm. Total of 10 scans were averaged for each measurement.

Raman Analysis

Raman data was conducted using a Bruker Senterra (Billerica, MA). Lyophilized samples were prepared by pressing the sample on SpectRIM steel slide. A total of 100 scans were collected in the 70-2740 cm^{-1} range with grating 1, Aperture 50 x and 1000 μm as optical filter setting. The excitation wave length was 532 nm with laser power set at 20 mW. GRAMS software was used to analyze the samples. Due to the large protein signal compared to weak buffer signal in the spectral regions of interest, background subtraction was not applied. The

data was processed using baseline subtraction, offset correction, and area normalization steps to enable ease of comparison of the different spectra.

RESULTS

Residual moisture and cake properties of lyophilized formulations

The final moisture content in lyophilized drug product formulations can significantly affect long-term protein stability of the protein. Increased moisture content can increase the deterioration rate of proteins and can facilitate crystallization of formulation excipients.[28] In our study, all cakes stored at 4 ºC had similar appearance with no visible signs of collapse. The final moisture level in all lyophilized samples was below the recommended 2% level, except for one sample stored at 4 ºC for 12 months. This sample had 2.46% residual water content (RWC) but also had no visible sign of collapse or stability issues post reconstitution (Table 2). There appeared to be little effect of residual moisture on the rate of aggregation during storage at 4 ºC for 12 months as discussed in next section. Also, no cake collapse was seen in most of vials even during prolonged storage at 50 ºC.

Table 2. Moisture content (RWC) measurement by Karl Fisher for lyophilized samples stored at 4ºC.

k''	RWC (%) 3 month	RWC (%) 6 month	RWC (%) 9 month	RWC (%) 12 month
STD (5% Lactose)	**5.11**	**5.33**	**4.91**	**5.34**
C3M_4ºC	0.45	0.07	0.15	0.98
C3MSu_4ºC	1.09	1.03	0.81	2.46
C5M_4ºC	0.50	0.20	0.75	0.84
C5MSu_4ºC	0.80	0.17	0.11	1.73
C7M_4ºC	0.26	0.15	0.02	0.79
C7MSu_4ºC	1.87	0.46	0.18	1.83

(STD=5% Reference Standard as control, C3M = 10mM sodium citrate, 4% mannitol, at pH3; C3MSu = 10mM sodium citrate, 2% sucrose, 4% mannitol, at pH 3; C5M = 10mM sodium citrate, 4% mannitol, at pH 5, C5MSu = 10mM sodium citrate, 2% sucrose, 4% mannitol at pH 5; C7M = 10mM sodium citrate, 4% mannitol, pH 7; C7MSu = 10mM sodium citrate, 2% sucrose, 4% mannitol at pH 7)

Physical and covalent stability on long term storage

All proteins stored at 4 ºC were clear upon reconstitution and no particulate matter was visible. The C3MSu formulation stored at 50 ºC for 6 months, however, appeared to undergo a color change, from clear to brown. It is known that at low pH sucrose may hydrolyze readily to form fructose and glucose, which can react with proteins via Millard reaction[8] resulting in the browning of the cake or the liquid. Although this reaction was suspected to have occurred, the color change was not examined further.

Size-exclusion chromatography (SE-HPLC) was used to assess the physical stability of proteins upon reconstitution with respect to aggregation during storage.

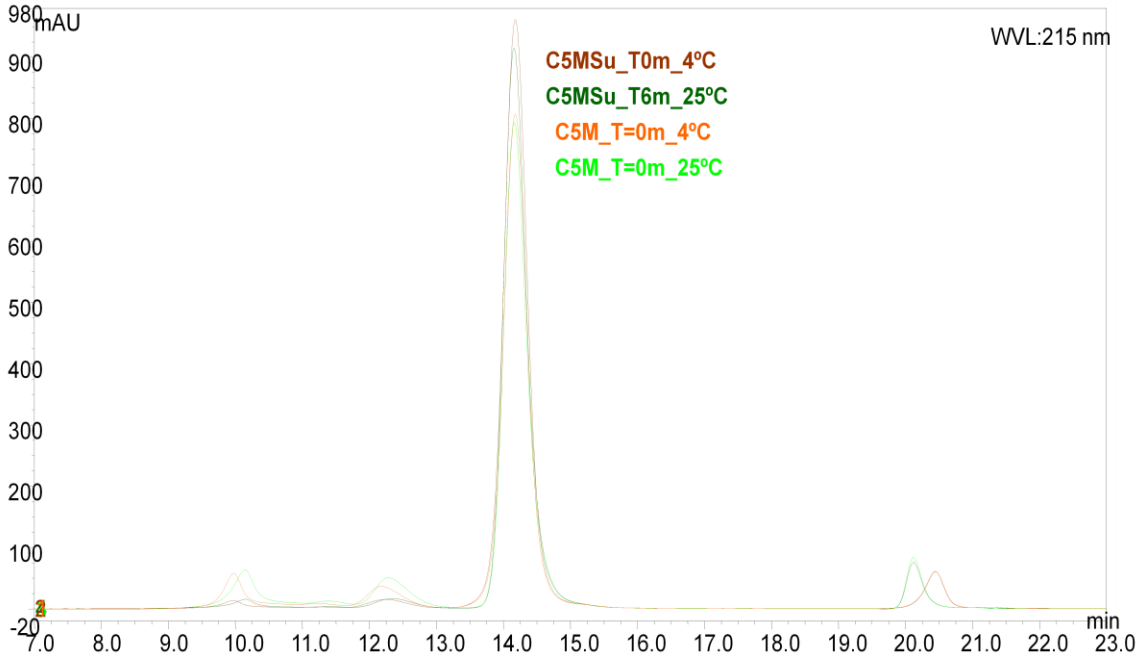
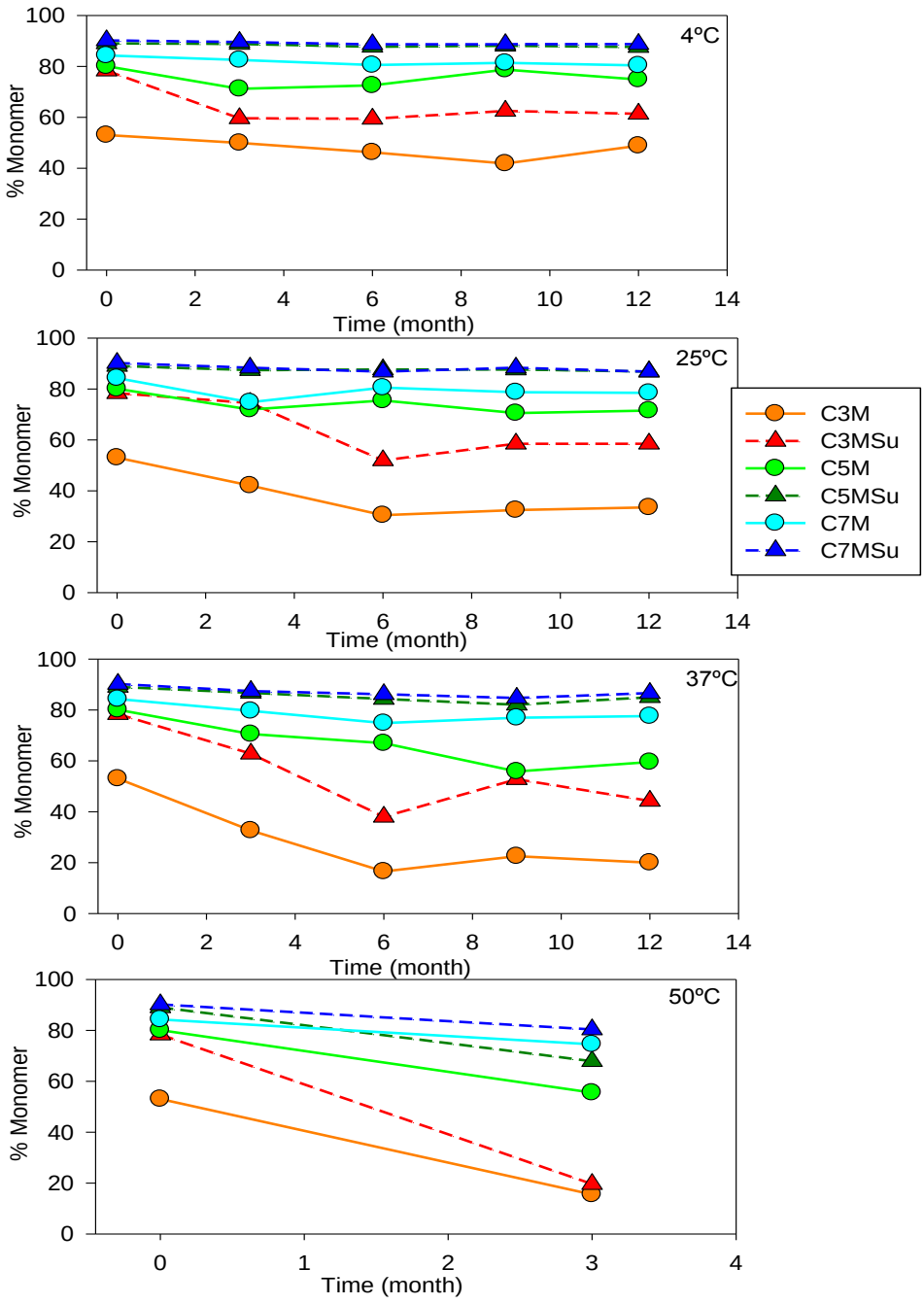


Figure 1. SE-HPLC chromatogram of anti-streptavidin IgG1 monoclonal antibody showing the different species

Loss of monomer was concomitant with increase of high molecular weight aggregates (Figure 1) and low molecular weight "clip" species, presumably resulting from backbone hydrolysis (Figure 1), were monitored as a function of time at different temperatures. It is well known that the pH of the protein solution before lyophilization often affects the stability of dried protein products during long-term storage.[28] It was observed that the loss of protein monomer (Figure 2) was mainly due to formation of high molecular weight aggregates at all pH conditions and, to lesser extent, clip species formation at pH 3 conditions data not shown. The presence of sucrose, however, inhibited the loss of monomer at the pH 3 condition. More than 45% and 30% loss of monomer were observed at time zero for C3M and C3MSu respectively. There was no significant difference for C5MSu and C7MSu formulations at the initial time point. Liquid samples stored at -80 ºC did not show any difference among the formulations along with time (Data not shown). Long-term protein storage at 2-8 ºC and higher temperatures was performed to further test protein stability. The amount of monomer decreased with time and increasing temperature for most of the formulations except for C5MSu and C7MSu. There were greater monomer losses for samples stored at higher temperatures over the period of one year (Figure 2). Overall, monomer loss was greatest in the C3M condition, followed by C3MSu, then C5M, and C7M. The concomitant increase of soluble aggregate was more for both C3M and C3MSu followed by C5M and C7M (data not shown). There was no difference observed for C5MSu and C7MSu formulations, and the C5MSu and C7MSu conditions were the most stable. Not only did freeze-drying with sucrose/mannitol result in greater retention of protein monomer compared to mannitol alone, but the presence of sucrose also improved stability at all pH conditions (Figure 2). This is not surprising, as sugars such as sucrose have been shown to protect proteins from time-dependent denaturation. [29]

A


4ºC
25ºC
37ºC
50ºC
% Monomer
Time (month)
C3M
C3MSu
C5M
C5MSu
C7M
C7MSu

B

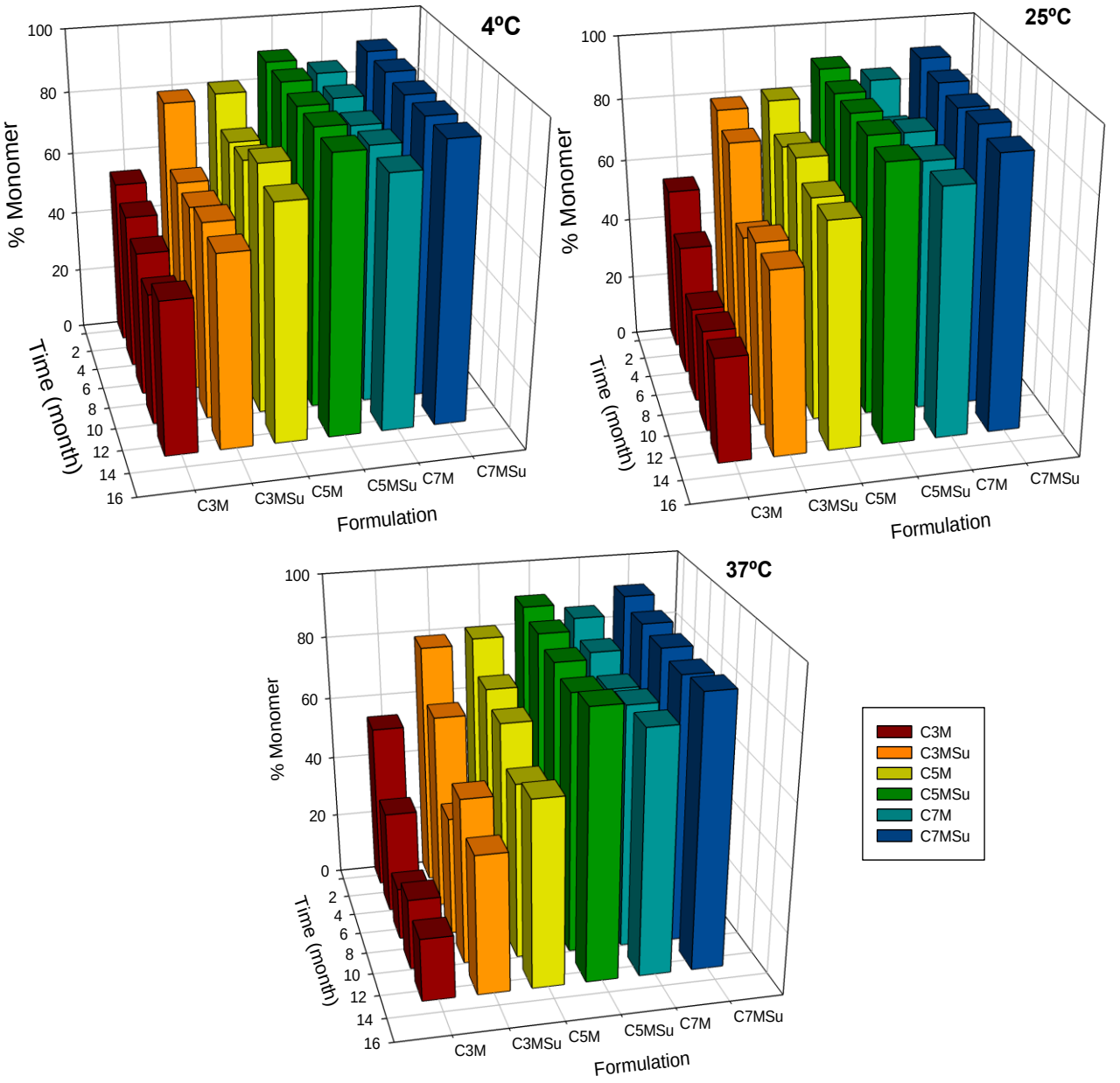


Figure 2. Stability data of 5mg/mL IgG1 lyophilized in different formulations at indicated accelerated storage conditions as measured by Size Exchange Chromatography (SEC). (A) Scatter plot of SEC data. (B) Different rendition of the same data as 3D bar plot comparing different formulation conditions.

Cation Exchange Chromatography (CEX)

Cation exchange chromatography was used to monitor charge related covalent changes happening in the mAb as a function of time, temperature and formulation conditions. The % CEX main peak as well as the % acidic species due to clips and isomerization and basic isoforms due to aggregation, deamidation and formation of succinimide were evaluated. A representative chromatogram (Figure 3) shows the changes in the CEX profile along with time of storage at 4 °C and 25 °C.

An increase in acidic peak was observed for low pH formulations due to clip formation. Overall post lyo samples did show decreased main peak at lower pH compared to higher pH formulation salso due to increase basic species. However, the presence of sucrose at pH 3 showed protection of protein against degradation and this condition showed no significant difference of % main peak recovery (Figure 4). Samples stored at 2-8 ºC for 1 year did not show significant changes in % main peak versus -80 °C samples except for pH 3 (Figure 4).

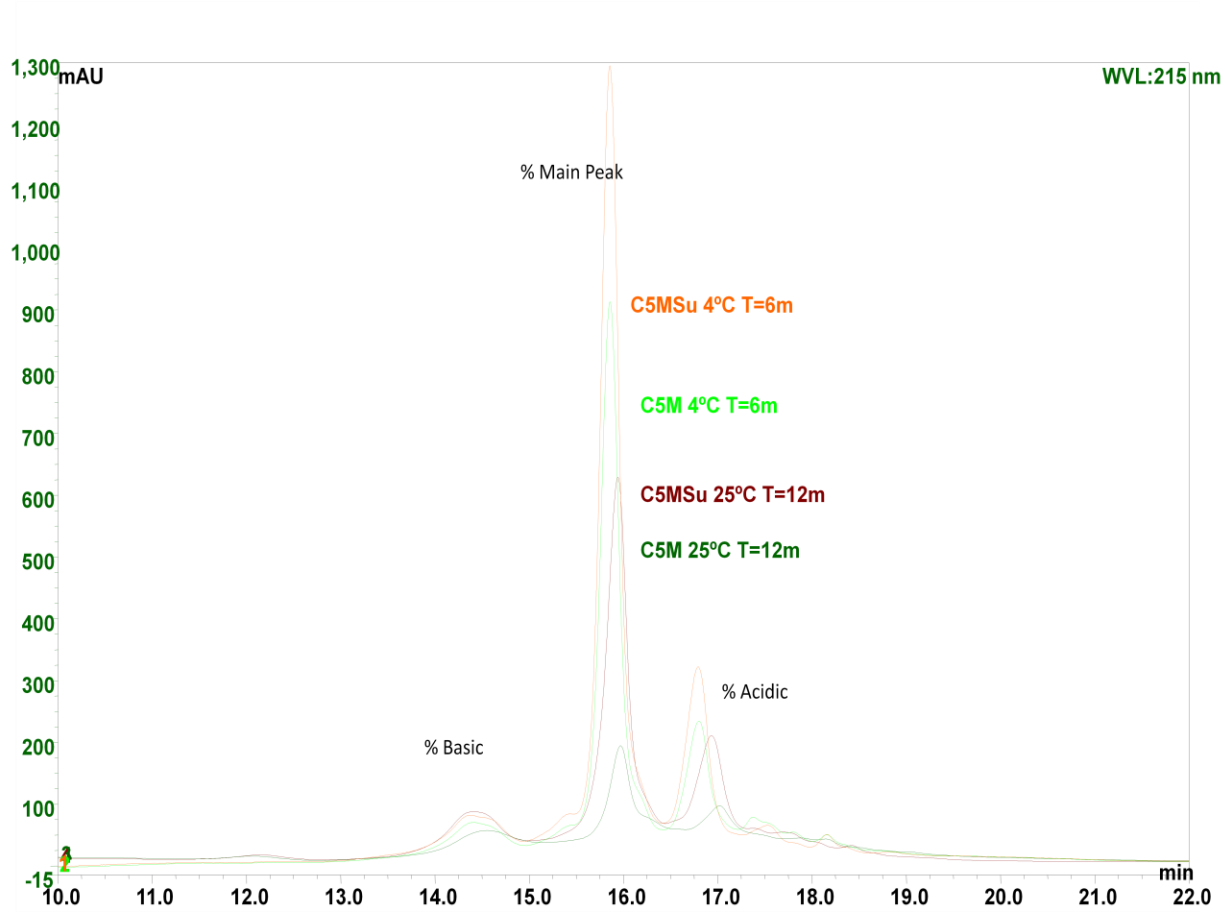


Figure 3. CEX chromatogram of anti-streptavidin IgG1 monoclonal antibody showing the main, acidic and basic forms.

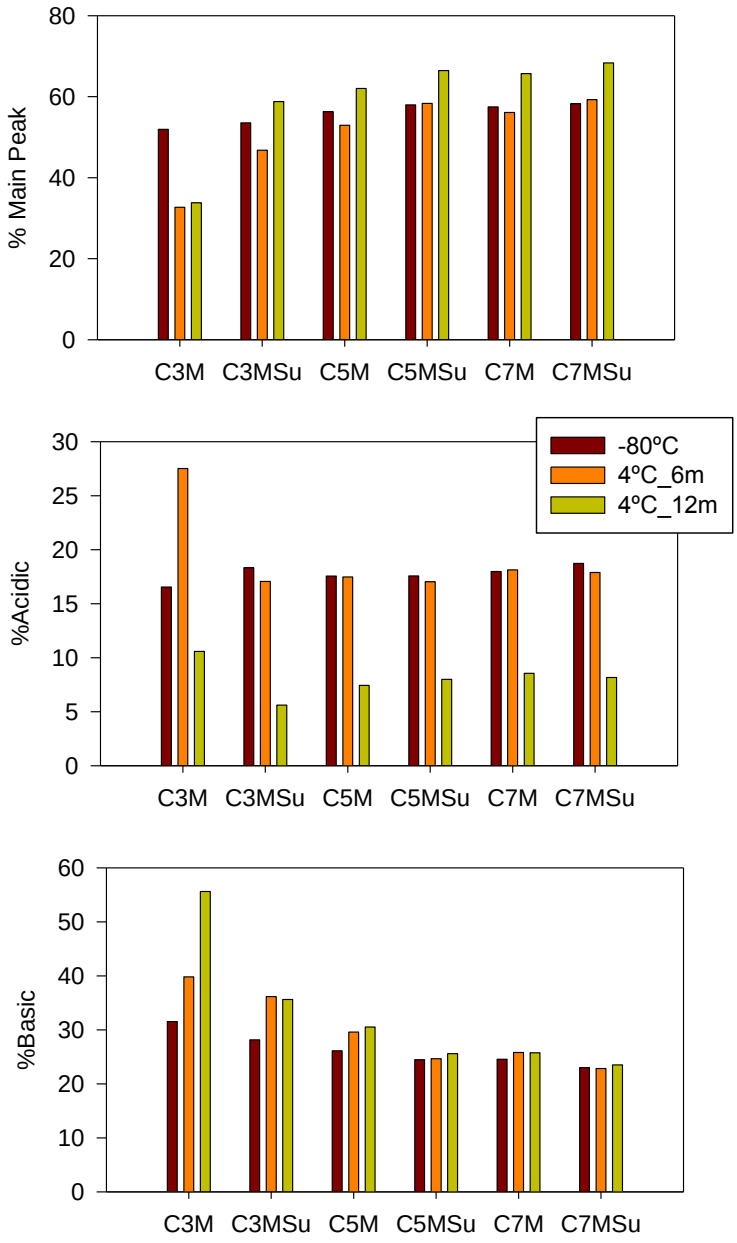


Figure 4. Stability data of 5mg/mL IgG1 lyophilized in different formulations at indicated accelerated storage conditions as measured by Cation Exchange Chromatography (CEX).

Sub-visible particles were measure by HIAC for 2 µm, 5 µm, 7.5 µm, 10 µm, 15 µm, 20 µm, and 25 µm particle size bins (Figure 5). The 2-micron particle numbers were all below 5000. Following C7M, C5M showed the highest cumulative counts of particles. It seems that higher pH formulations produced more particles than lower pH formulations. At pH 7, the addition of sucrose helped to reduce the numbers of sub-visible particles. Also, at pH 5 the sucrose/mannitol formulation had lower numbers of particles than formulations containing only mannitol. Conversely, samples formulated at pH 3 did not show any difference between samples with and without sucrose. Particles greater than 2-micron represent protein stability and demonstrate overall particle trend/stability. Most of the formulations had particle counts below the USP guidelines (≤6000 particles per container for particles 10 µm and ≤600 particles per container for particles ≥25 µm)[30] for all formulations except for C7M conditions which were above these limits.

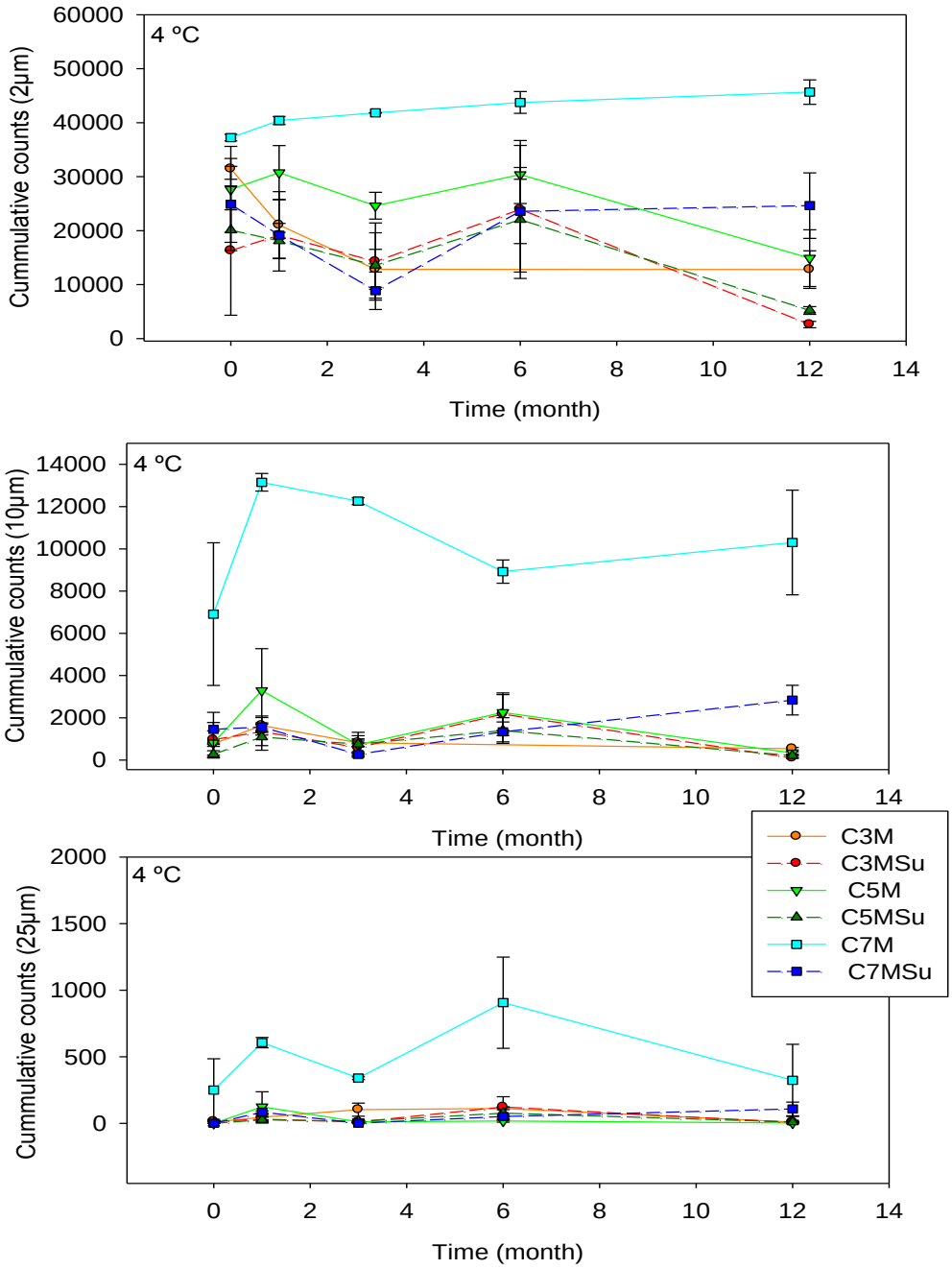


Figure 5. Stability data of 5 mg/mL IgG1 lyo in different formulations at indicated accelerated storage conditions as measured by sub-visible particle by HIAC. 2 µm, 10 µm, and 25 µm cumulative counts per mL at 4 °C.

Effect of Lyophilization storage in the solid state on the secondary structure of IgG1 as measured by FTIR and Raman analysis

In our investigation, all FTIR data were area normalized and base line corrected, and the second derivative was obtained over the amide I region (1600-1700 cm^{-1}). The amide I band (amide C=O stretching) in this spectral region is commonly used to obtain protein structural information,[4] as it is influenced by hydrogen bonding, which reflects the variation in secondary structure of the protein.[8] The second derivative spectrum of hydrated native IgG1 antibody had two dominant bands at 1640 cm^{-1} and 1690 cm^{-1}. The bands at 1640 cm^{-1} are due to the native intramolecular β-sheet structure, and bands at 1615 cm^{-1} and 1690 cm^{-1} are due intermolecular H-bonded anti-parallel β-sheet, which in this system is representative of aggregates in the solution state.[31]

The band at 1640 cm^{-1} is a marker band for native-like structure. Thus, a more intense band at this position means a higher degree of native structure in the sample.[6] The band positions agree well with those reported for general IgG1 molecules in general, as antibodies are mainly composed of intramolecular β-sheet structures with a small portion of α-helix.[32] It is expected that the lyophilized formulation, which has protein spectra similar to the native protein in the liquid state, should have better storage stability.[33]

First, the spectra of each hydrated formulation were compared with the goal of determining quantitatively the degree of retention of native structure (Figure 6). It was observed that the pH 3 conditions has the lowest intensity of the 1638 cm^{-1} band compared to other pH conditions, indicating loss of native β-sheet structure occurred (Figure 6). Upon drying the IgG1 solution, slight conformational changes were observed. The β-sheet band shifted to 1640 cm^{-1}, became broader, and the intensity decreased as a result of freeze-drying stresses (Figure 6). It was observed that immediately after lyophilization, pH 5 and sucrose-containing formulations

retain most of their native structure, whereas pH 3 and pH 7 formulations do not. More native structure is retained in the pH 7 formulation compared to pH 3. Addition of sucrose to pH 3 and pH 7 conditions does help maintain native secondary structure in the solid state compared to the same pH formulations lacking the stabilizers.

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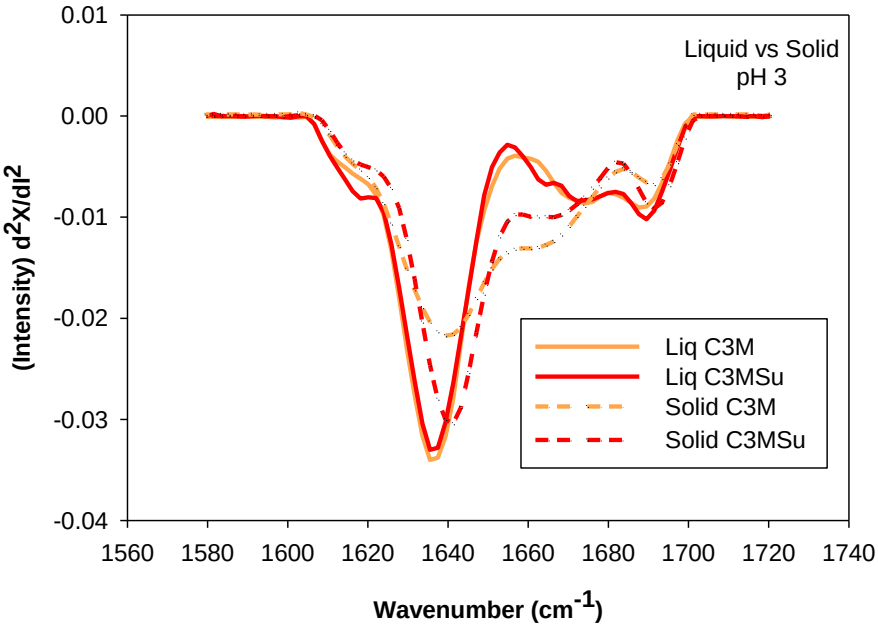

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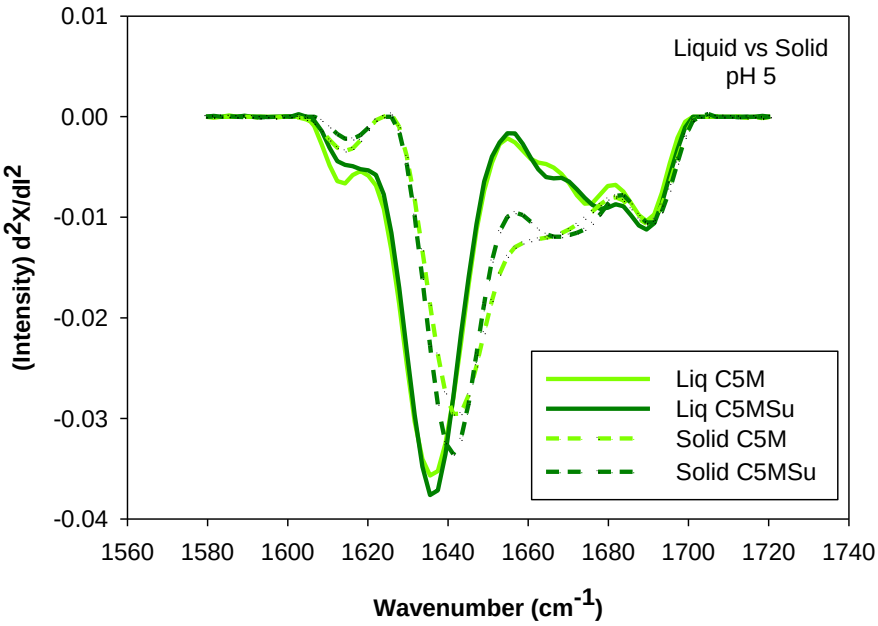

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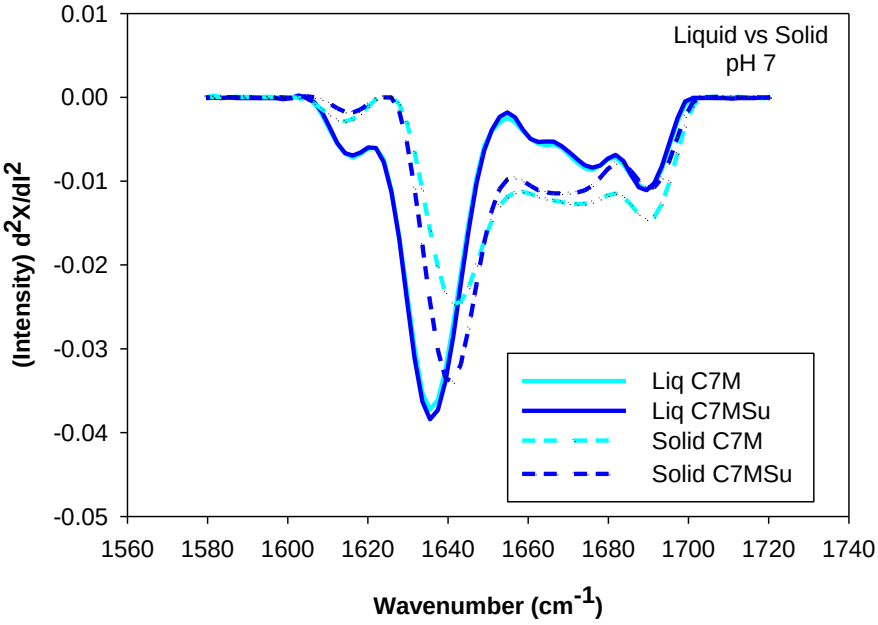

Figure 6. Comparison of secondary derivative FTIR spectra of IgG1 in aqueous solution and solid, dried formulations at T=0 month in different formulations. (A) pH 3, (B) pH 5, (C) pH 7

All formulations demonstrated a decrease in second derivative spectral intensity at 1640 cm^{-1} with time at all temperatures (Figure 7, Figure 8). Also, the band intensity at high frequency 1690 cm^{-1} decreased, indicating a loss of β-turn with time. The spectrum showed a general broadening of the peaks with time, which likely indicates chain disordering or loosening of structure.

Samples at pH 3 showed the greatest intensity loss when compared to other formulations. On the other hand, samples formulated at pH 5 and pH 7 maintained higher intensity and showed less change when compared to pH 3. Also, samples formulated with sucrose displayed higher intensity than samples formulated without sucrose.

C3M displayed the lowest intensity with time. At 12 months, a shift of β-sheet structure is observed for the C5M and C7M formulations. C5MSu and C7MSu maintained most of their native structure. The FTIR stability data at 25 ºC, 37 ºC showed similar trends as the 4 ºC samples (Figure 8). Overall, sucrose formulated protein at pH 5 retained more native-like structure than that at pH 7. These data together indicate that sucrose improves the structural stability of this IgG1 regardless of pH, but that native-like structure is best preserved at pH 5.

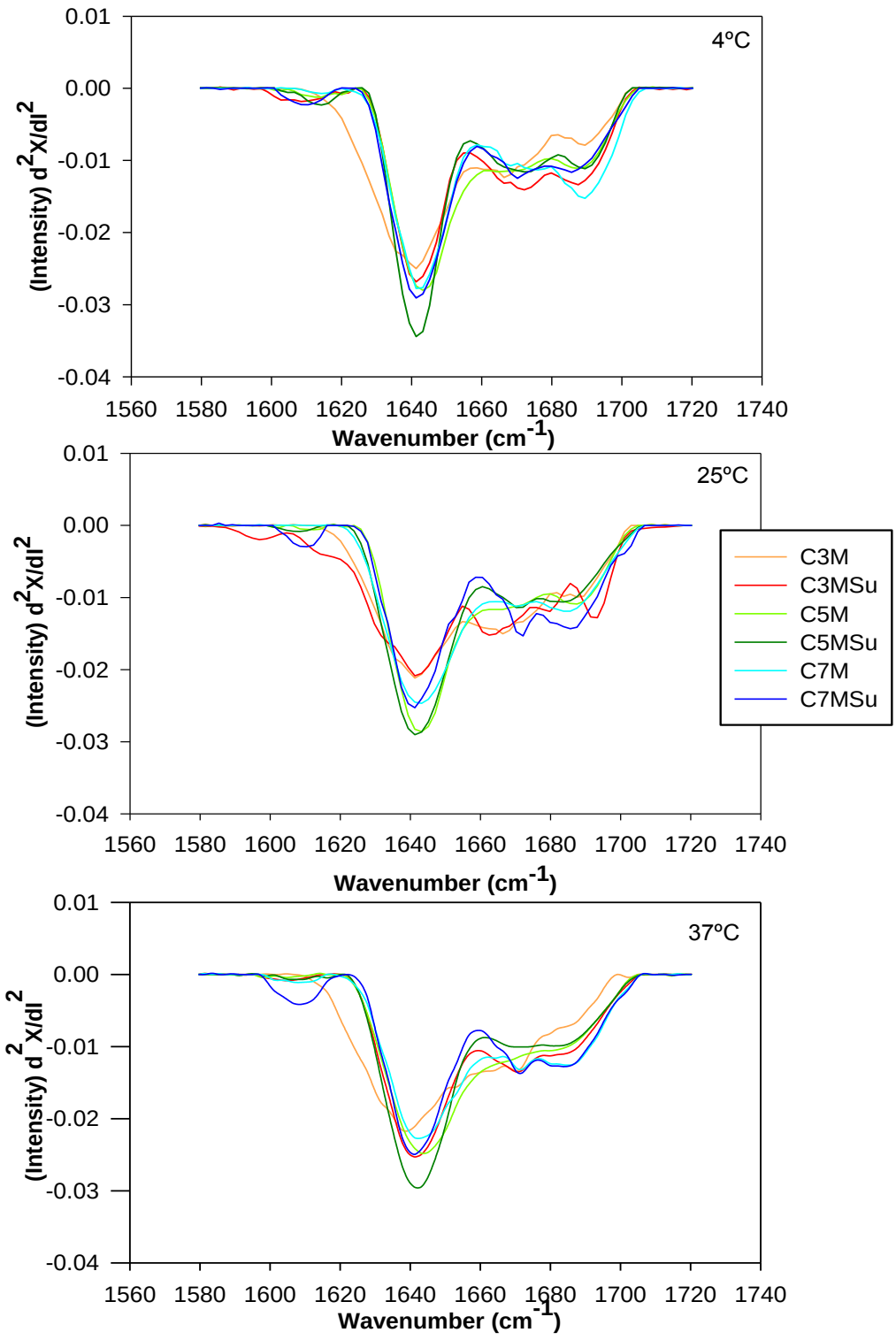

Figure 7. Secondary derivative FTIR spectra of solid protein at 4 °C, 25 °C and 37 °C at T=1m

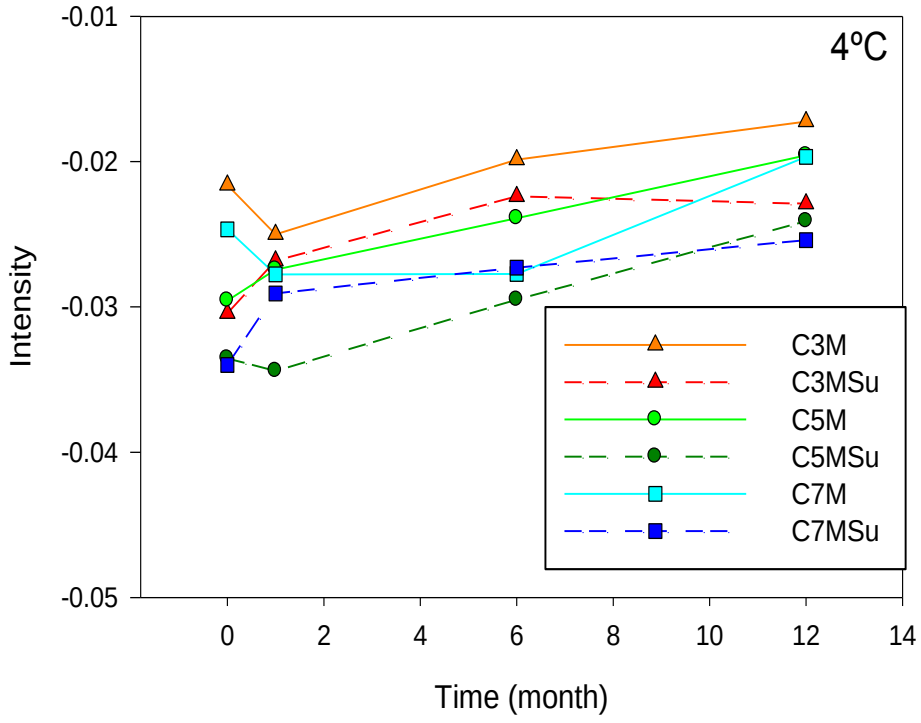

Figure 8. Second derivative FTIR spectra of IgG1 formulations stored at 4 ºC. The intensity at 1640 cm^{-1} is plotted as a function of time.

Figure 9 shows the relationship between the amount of monomer lost as observed using SE-HPLC (upon reconstitution) and percent native β-sheet intensity change in solid state for the different protein formulations at different time points.. The percent change in monomer was calculated by comparing stability at 4 °C, 25 °C, and 37 °C recorded at different time points post-reconstitution compared to liquid state at time 0 (pre-lyo control)**.** Similarly, percent change in native β-sheet intensity in solid state was calculated by comparing the structure in solid state stability samples at different temperature and time points versus liquid state at time 0 (pre-lyo control). β-sheet intensity change was less for samples containing sucrose/mannitol. There was a correlation between the degree of change in monomer loss and loss of percent native β-sheet intensity for each formulation (Figure 9). The maximum loss of monomer was observed

for pH 3 condition by SE-HPLC. This was due to clip formation at pH 3 conditions. Thus, the changes of monomer loss when compared to time zero were greatest for samples formulated at pH 3. There was greater change of monomer and percent β-sheet intensity change for formulations at pH 3 compared to pH 5 and pH 7 and with increasing temperature. The overall correlation plots showed r^2 value of 0.78 for 4 ºC storage, 0.44 for 25 ºC storage, and 0.55 for 37 ºC.

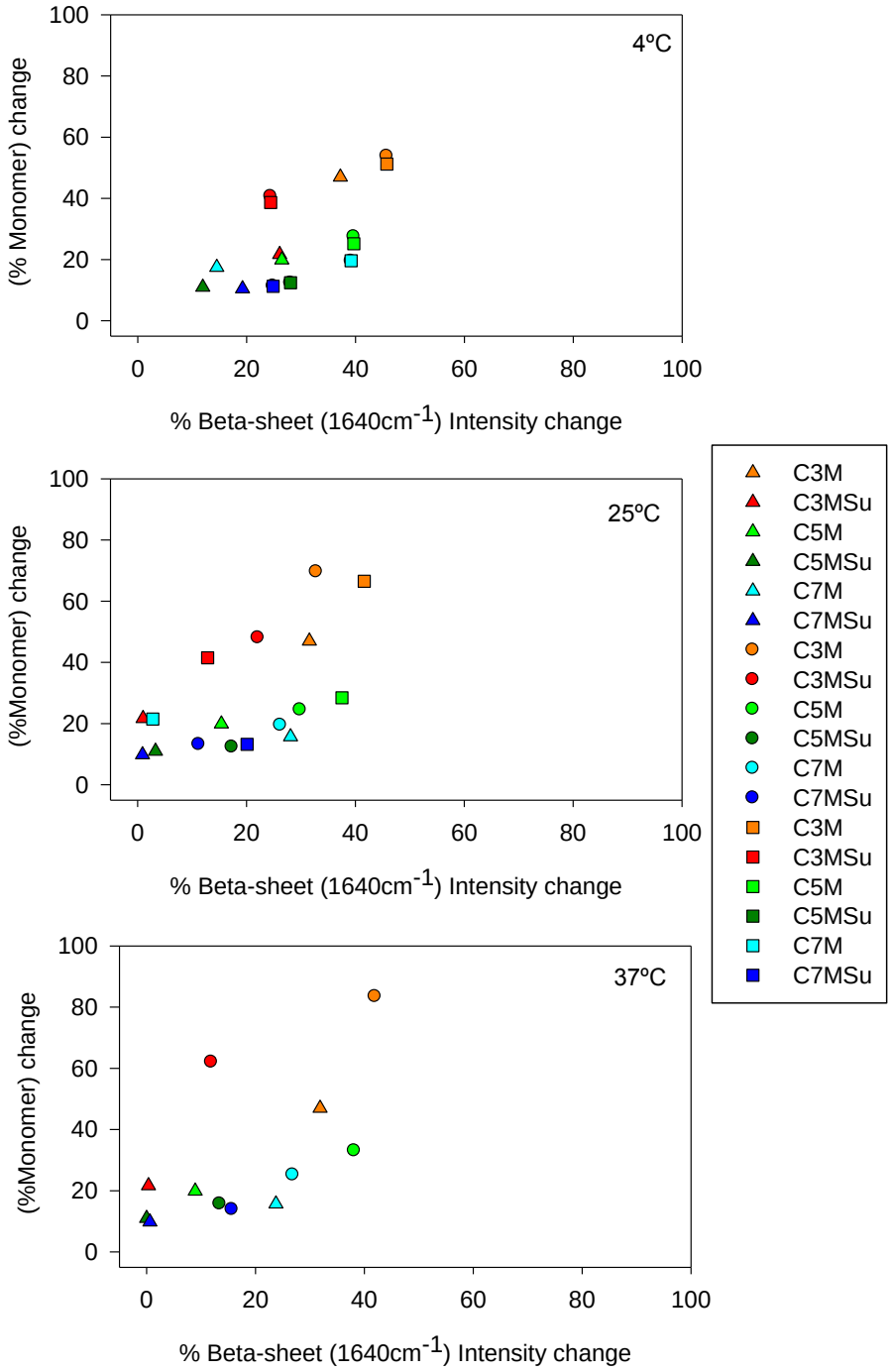

Figure 9. Correlation plot showing percent monomer change after reconstitution as measured by SE-HPLC versus % native β-sheet intensity change in solid state △: sample pulled at 0 month time point. ○: sample pulled at 6 month time point. □: sample pulled at 12 month time point.

Tertiary structure of protein by solid-state fluorescence

The maximum fluorescence intensity observed in the emission scan was at 329-321 nm (Figure 10). Typically, this spectral feature specific to tryptophan emission denotes that tryptophan is buried inside the protein in a hydrophobic environment.[34] No shifts to lower or higher wavelength were detected, but changes in intensity at the wavelength maxima were observed under all formulation conditions along with temperature and time.

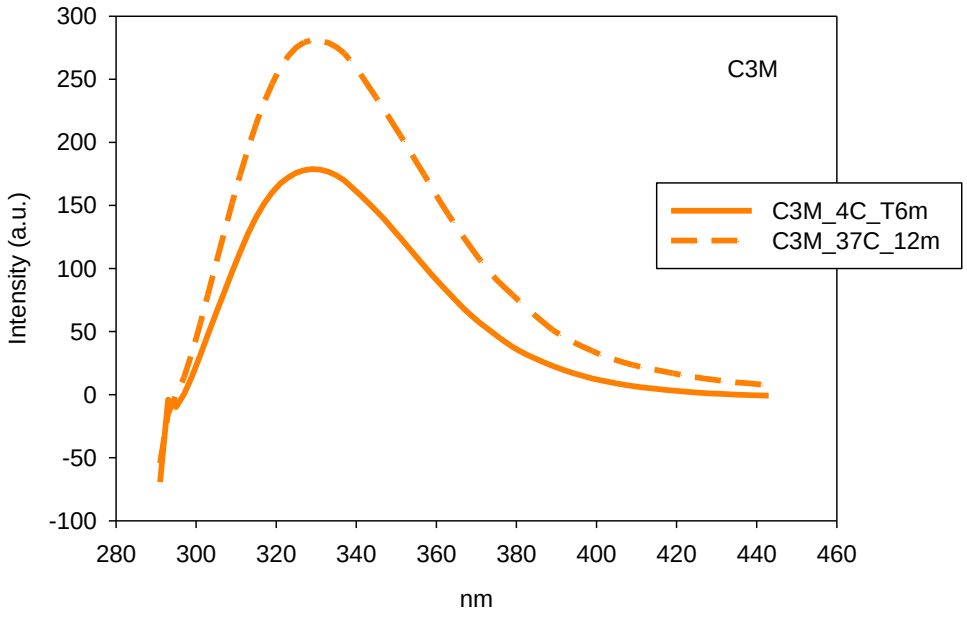

Figure 10. Example of anti-streptavidin IgG1 monoclonal antibody solid-state fluorescence profile.

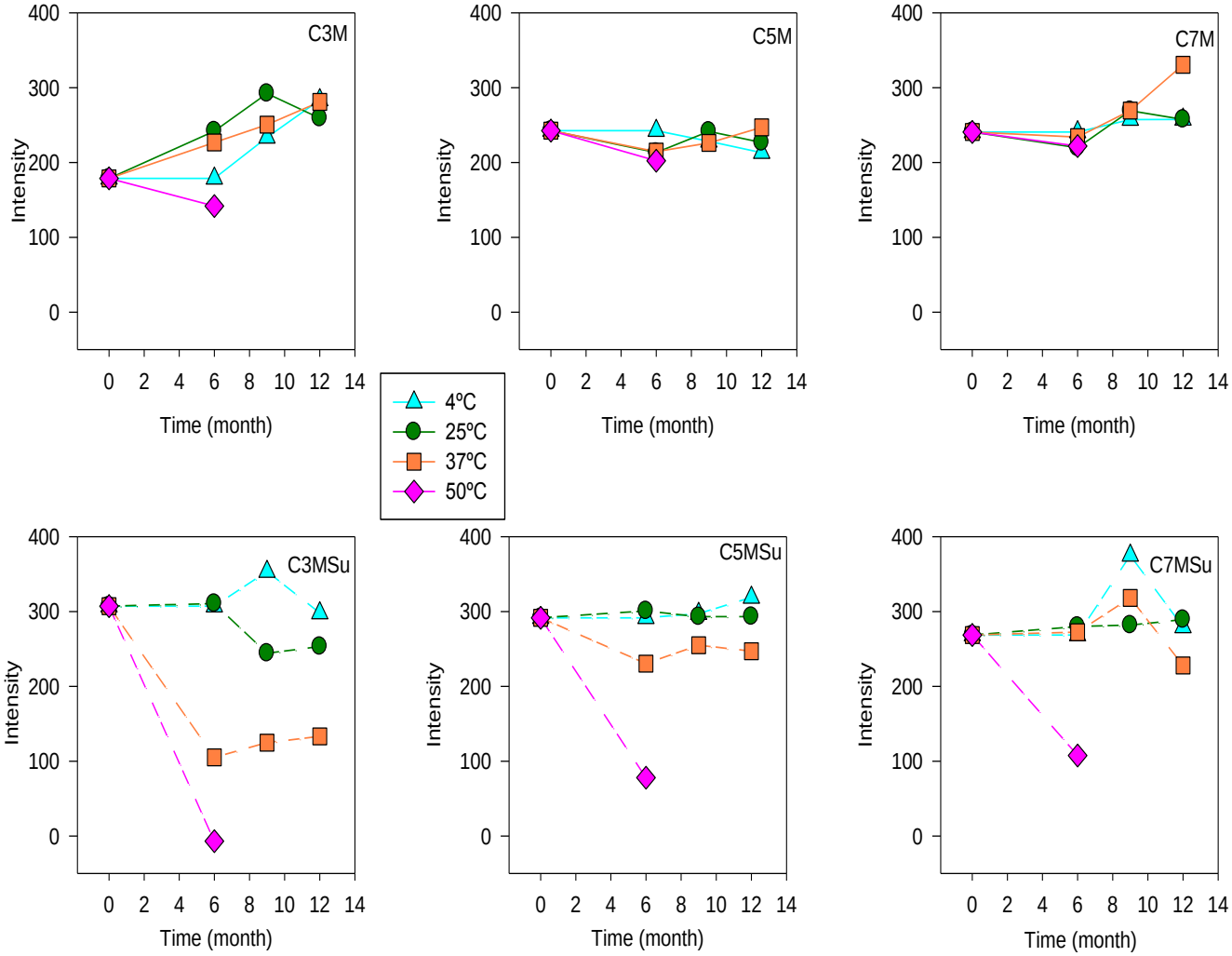

Figure 11. Solid-state fluorescence data. The maximum fluorescence intensity at 329 nm is plotted versus time (months).

Figure 11 shows the intensity of the intrinsic fluorescence spectra of lyophilized proteins in their respective buffers in the solid state with time. The effect of storage temperature was not different for the mannitol only formulations. Mannitol containing formulations showed some intensity changes at pH 3. However, protein at pH 5 and pH 7 did not show differences in their fluorescence intensity with varied temperature and over the period of 12 months. For sucrose formulations, samples stored at higher temperatures had lower intensities at pH 3 and pH 5. By

comparison, there was more change in intensity observed at different temperatures for C3MSu. C5MSu intensity also changed at different temperatures and these changes were not significant for C7MSu. Fluorescence intensity of sucrose containing formulations showed an decrease with increasing temperature and time rather than vice versa observed for mannitol alone formulations.

Figure 12 shows the relationship between the amount of monomer lost as observed using SE-HPLC (upon reconstitution) and solid-state fluorescence intensity change at 25 °C, and 37 °C (versus 4 °C) recorded at different time points. The percent change in monomer was calculated as described in the FTIR section. Percent change in absolute value of fluorescence intensity in solid state was calculated by comparing the intensity in solid state stability samples at 25 °C and 37 °C at different time points versus corresponding intensity of 4 °C samples. The maximum loss of monomer was observed for pH 3 condition by SE-HPLC. The changes of monomer loss when compared to time zero were greatest for samples formulated at pH 3. At 4 °C, only C3M condition showed greatest fluorescence intensity changes. The degree of change in monomer loss and fluorescence intensity for each formulation increased with increasing temperature (Figure 12). There was greater change of monomer for formulations at pH 3 compared to pH 5 and pH 7 compared to fluorescence change. These results suggested that there may be some changes in the conformation of the monomer occur around the environment of the tryptophan at pH 3 more than at the higher pH conditions. The increased degree of change may signify heterogeneity of tryptophan environment in the native to unfolded state ensemble of IgG1 that may in turn translate to the increase level of aggregates on reconstitution of the lyophilized samples at pH 3 condition. The overall correlation plots showed r^2 value of 0.56 for 4 ºC storage, 0.70 for 25 ºC storage, and 0.39 for 37 ºC.

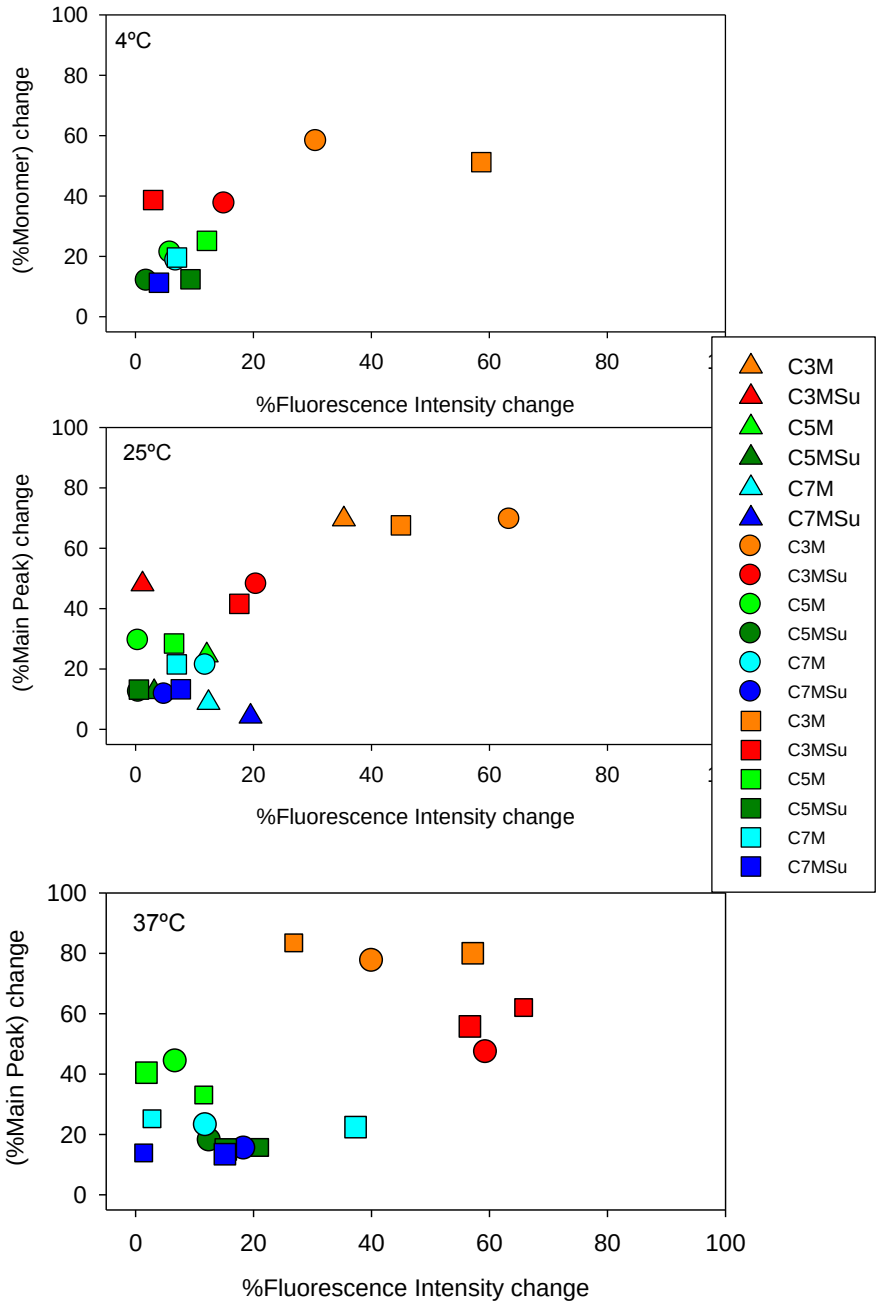

Figure 12. Percent monomer change after reconstitution as measured by SE-HPLC versus % absolute solid-state fluorescence intensity change at 25 °C and 37 °C versus 4 °C for each IgG1 lyophilized formulation. △: sample pulled at 6 month time point. ○: sample pulled at 9 month time point. □: sample pulled at 12 month time point.

Secondary and tertiary structural changes as monitored by Raman spectroscopy

The amide I band from the Raman spectra was used to characterize the secondary structural changes in the protein and as orthogonal technique to FTIR for tracking secondary structural changes in the solid state.

Raman spectra in the 1500-1800 cm^{-1} region for lyophilized protein is shown in (Figure 13).

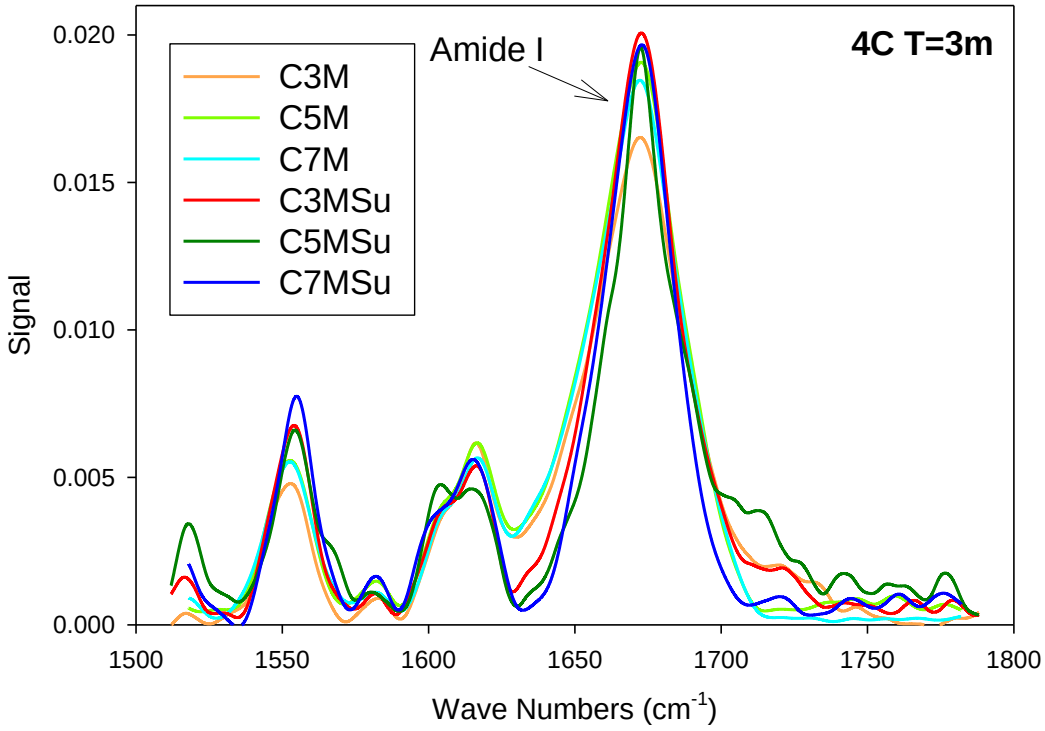

Figure 13. Raman spectra at amide I region of IgG1 lyophilized formulation at 4 °C.

The major band in the 1630-1720 cm^{-1} region is the amide I band, which is used to monitor secondary structure of the protein. The band at ~ 1670 cm^{-1} is characteristic of high β-sheet containing proteins. Samples formulated with mannitol only (C3M, C5M, and C7M) had broader bands than sucrose formulations (C3MSu, C5MSu, and C7MSu) (Figure 13). The

broader amide I peaks on the higher wave number side are usually associated with increased turn and unordered contents.[48] In addition, C3M had the lowest peak intensity followed by C7M, and then C5M at ~1670 cm-[1] region. The sucrose-containing formulations had higher intensity peaks than the mannitol only formulation.

The peaks in the 1550-1650 cm-1 region are due to the aromatic side chains (Figure 14). The 1615 cm^{-1}, intensity peak is associated with tryptophan, tyrosine, and phenylalanine. C5MSu condition showed the lowest intensity band for, and the highest intensity bands were observed for C3M and C5M. The 1604 cm^{-1} band is associated with phenylalanine, and was most prominent for C5MSu formulation. The other formulations showed a decrease in peak intensity at 1604 cm^{-1} and in most cases showed a mere shoulder.

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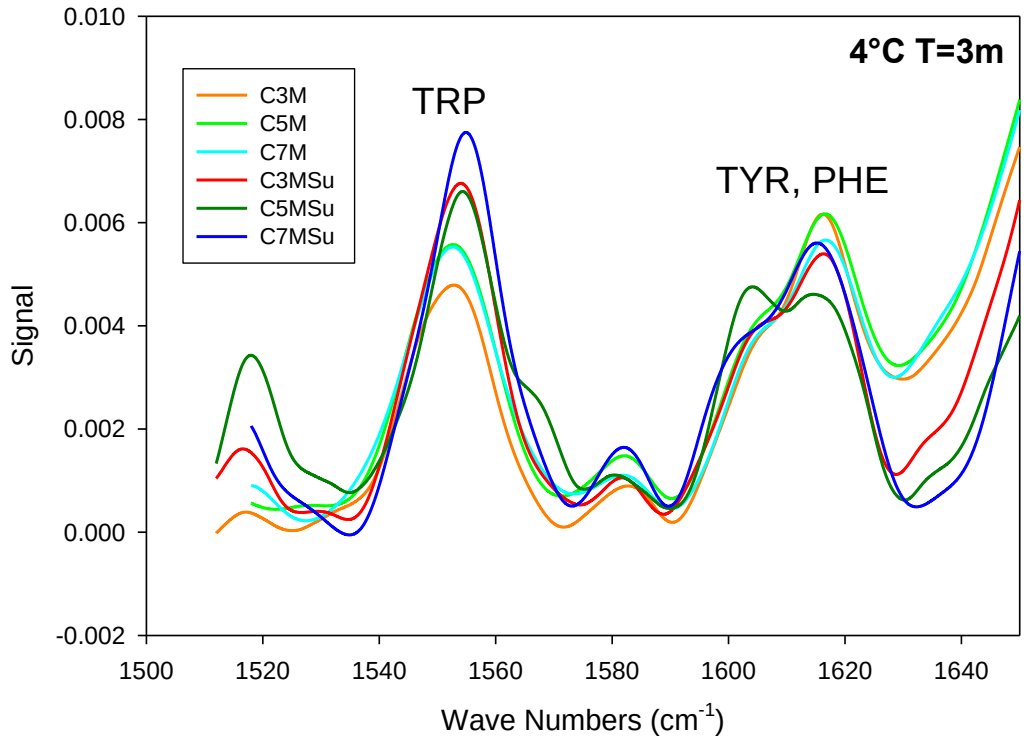

Figure 14. Raman spectra of tryptophan region of IgG1 lyophilized formulation at 4 ºC at T=3m

Raman spectral peaks for mannitol formulations were less intense and shifted to 1552 cm^{-1}, whereas the sucrose containing formulations showed compact peaks at 1554 cm^{-1}. This shift is associated with ring vibrations due to the tryptophan. [35,36,37,38,39]

At 4 °C, C5MSu showed the least intensity at 761 cm^{-1}, and C3M the greatest (Figure 15). This region represents the cation-π interactions that are intrinsic to protein's structure and likely contribute to protein stability.[40]At 37 °C, a shift was observed for C3M and C3MSu. C5MSu showed the greatest intensity for this band (data not shown).

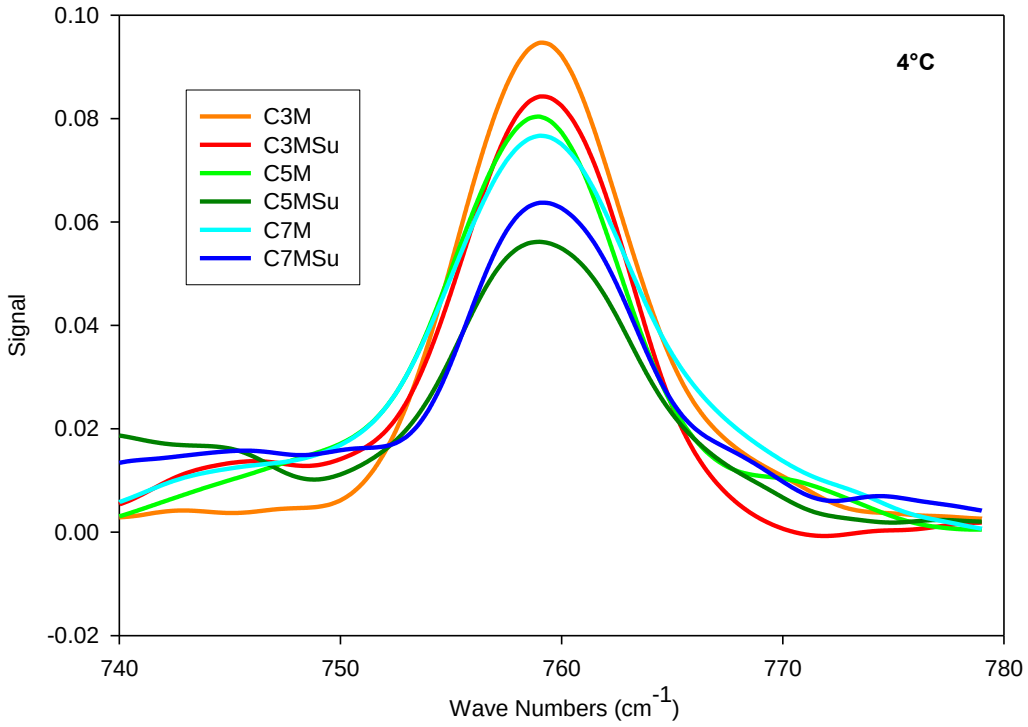

Figure 15. Raman spectra of cation-π interaction region of IgG1 lyophilized formulation at 4 °C at T=3m

The amide I peak comparison at 37 °C result was consistent with the results at 4°C. Samples formulated with mannitol only had broader bands than sucrose formulations (Data not

shown). The absence of sucrose led to significant loss in the β-sheet in the amide I region. C7M sample had the lowest intensity peak followed by C3M and then C5M.

The difference between the absence and presence of sucrose in the formulations was apparent and noticeable in the tryptophan peak at 1554 cm^{-1} at 4 °C. However, the difference was not obvious in samples stored at 37 °C. Peaks were all grouped together with the exception of C3M. The intensity of this peak in the C3M sample was the least. In the C3MSu spectrum this peak had the highest intensity of all the formulations.

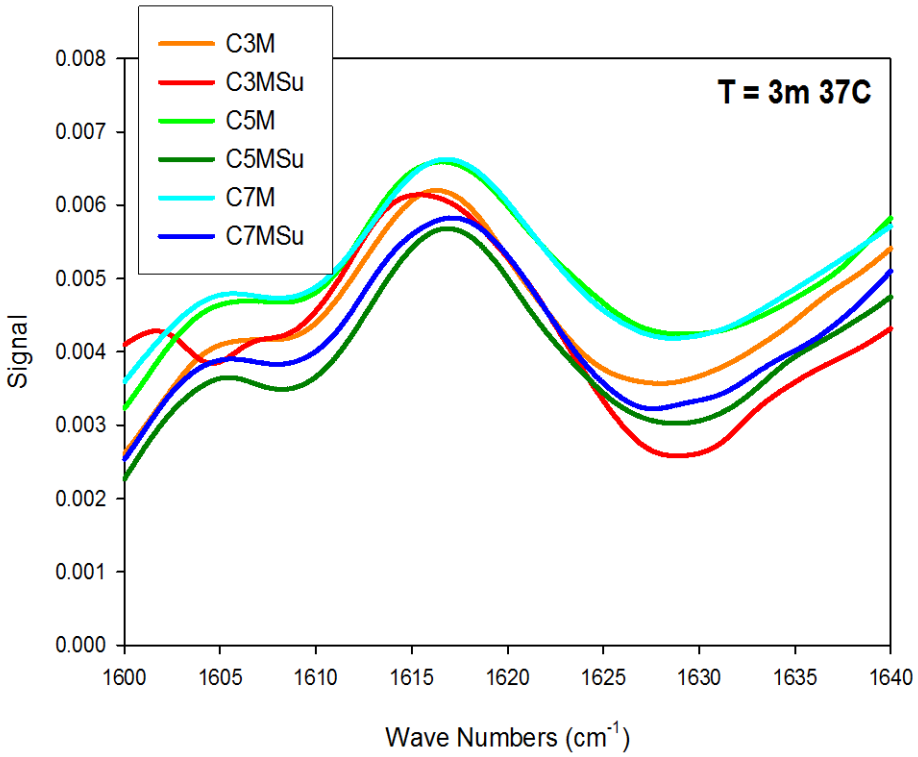

Figure 16. Raman spectra at 1604 cm^{-1} region of IgG1 lyophilized formulation at 37 °C at T=3m

There was no obvious correlation for 1604 cm^{-1} band at 37 °C (Figure 16) with 4 °C data. Furthermore, the C5MSu peak resolution at 4 °C samples was lost with 37 °C storage. In addition, at 37 °C, the C5MSu showed the lowest band at 1615 cm^{-1}, and C7M and C5M as the

band with the sharpest peak. In literature, the basis for Raman spectral changes especially for monitoring tertiary structure have not been clearly understood compared to the other techniques (e.g. spectral changes for FTIR). The ability to use Raman spectra changes to monitor protein stability has to be explored in more detail through a different investigation outside the scope of this thesis work. Correlation plots were not calculated for raman spectral intensity changes in the solid state versus loss of monomer by SE-HPLC (upon reconstitution) due to lack of enough data points as only time zero and time three month data points were available for comparison. However, it should be noted that the different Raman spectral regions of interest did show a trend with respect to intensity changes that was similar to ranking of formulations based on physical stability.

DISCUSSION

Effect of pH and stabilizer on solid-state stability

Numerous studies have emphasized the importance of the physical state of buffer and stabilizer components in stabilizing proteins during freeze-drying.[24] Generally, a protein with native conformation is expected to be more resistant to degradation during storage.[41] It was found that there is an impact of pH on the stability and activity of proteins. As such, possible changes in the acid-base characteristics of proteins should be considered since it can lead to destabilization of native structure during dehydration[42] affecting both physical and chemical stability. Proteins are often stable within a narrow pH range.[31] It was found that at extreme pH values proteins have tendencies to unfold and likely increase the amount of aggregates.[42]

In our stability study, the pH 3 formulation showed significant loss of monomer followed by pH 7, while pH 5 had the least monomer loss. Anti-streptavidin IgG1 has a pI of 8.8. It is well known that when the pH is close to protein's pI, protomers may associate, aggregate and form

particles.[43] Although pH 5 and pH 7 showed similar stability data by SE-HPLC, more sub-visible particles were detected in pH 7 formulations, particularly C7M, which had the highest sub-visible particle count. It is observed that pH plays a dominant role in determining the physical stability of the IgG1 in the lyophilized state.

Results for the proteins that were freeze-dried only in the presence of mannitol showed higher amount of aggregation. Mannitol is often used as a bulking agent in preparing lyophilized proteins, and it has been shown to crystallize upon freezing.[24] Crystallization is thought to remove mannitol from the protein phase and result in the loss of molecular interaction with proteins.[44] As such, maintenance of the amorphous state is important for imparting stabilization to proteins during storage.[42, 45] Inclusion of mannitol typically results in excellent cake structure, but proteins derive no protection from crystalline mannitol.[46] Mannitol provides some protection if it remains in the amorphous form.[4] Several studies have shown that mannitol when combined with amorphous stabilizers such as sucrose improves protein stability after lyophilization. These studies suggest that mannitol either facilitates sucrose-based stabilization of the protein or it directly stabilizes the protein when maintained in the protein phase during drying (amorphous form).[4] On the other hand, it is well known that sucrose assists in maintaining the native structure of proteins in the dried state.[25] The amount of monomer decreased with time and increasing temperature for most of the formulations except for C5MSu and C7MSu. Our SE-HPLC data and particle analysis data for anti-streptavidin IgG1 show that under the optimal pH condition, the presence of sucrose drastically improves structural stability and mannitol alone is less effective at preventing aggregation. The above findings reiterate the observations reported in the literature.[6,7]

Effect of pH and stabilizer on antibody secondary and tertiary structure in solid-state

A comparison of the results of the stability studies and spectroscopic analyses indicate a modest correlation exists between the retention of native structure in the lyophilized state and long term stability. The protein conformation was very sensitive to the pH of the formulation. Different pH spectra showed relative peak intensity differences as well as loss and gain of spectral features, which were indicative of conformational changes. Broadening of the individual amide I components in the spectra observed upon dehydration is indicative of general disordering of the protein backbone.[24] Aggregates in lyophilized IgG1 were detected by the reduction in the intensity of the native β-sheet at 1640 cm^{-1} as well as the increase in the area of the intermolecular band at 1620 cm^{-1} and 1690 cm^{-1}. [21, 25]

Based on the spectra, pH 3 was drastically altered compared to pH 5 and pH 7. A new, strong band was observable in the pH 3 formulations at 1615 cm^{-1}, indicating formation of a non-native structure during dehydration. This band and the enhanced absorbance near 1690 cm^{-1} are most likely indicative of a β-sheet structure formed by unfolding and aggregation of the protein during lyophilization.[47] Samples formulated at pH 5 and 7 had greater intensity bands at 1640 cm^{-1} region, indicating they retained their native structure.

The second derivative FTIR spectra acquired on samples stored at 4 ºC indicated a decrease in intensity of the band at 1640 cm^{-1} with time. Samples formulated at pH 3 displayed the most extensive structural changes. Similar trends were observed for samples stored at 25 ºC and 37 ºC. Greater loss of intramolecular β-structure was observed at higher temperatures for all samples.

In the absence of sucrose, the protein structure was perturbed dramatically, as indicated by a large decrease in the intensity of the band at 1640 cm^{-1}. The FTIR studies have shown that

many proteins undergo denaturation, often forming intermolecular β-sheet structures, upon lyophilization in the absence of stabilizers.[48] With the addition of sucrose into the formulation, the extent of perturbation was moderated, as the protein retained more "native-like" structure with sucrose in the formulation. It is well known that sucrose assists in maintaining the native structure of proteins in the dried state.[25] Freeze drying our IgG1 in the presence of sugars improved stability, resulting in spectra that are more native-like than in the absence of the sugar.[25] The addition of sucrose maintained native structure better than mannitol only formulations at each pH. Both stability data with SE-HPLC and FTIR indicated that samples formulated at pH 3 did not maintain the native structure. C5MSu, C7M, and C7MSu showed the greatest ability to maintain the sharp β-sheet band. The native β-sheet band intensity loss showed a general correlation with monomer loss with time (SE-HPLC).

Previous studies showed a correlation between fluorescence intensity and aggregation, [26] which resulted from changes in the tertiary structure that may open up certain hydrophobic pockets.[49] In our study, it was observed that formulation matrix as such can have an effect on the solid-state fluorescence spectra due to the effect of the excipients on fluorescence signal. The degree of intensity change does correlate with the long term stability. As observed from Figure 6 and 7, higher temperatures and longer time points show more tertiary structure change for pH 3 compared to other pH conditions. Also, the pH 3 conditions, showed the highest loss of monomer over time and the loss increased with temperature in the solid state. The addition of sucrose decreased the amount of monomer loss and also the magnitude of intensity change on the fluorescence spectra the pH 3 condition. This may be due to sucrose inhibiting heterogeneous conformational change of the IgG1 at pH 3 (shown in Figure 12) which may be the reason for protection against physical instability at this pH condition. At pH values of 5 and above, the

fluorescence change was not significant at 4 °C storage and correlated in general with lower loss of monomer peak.

In addition, Raman provided valuable information about conformational changes resulting from freeze-drying. Raman spectra provide information about both protein backbone and the orientation of the amino acid side-chains phenylalanine, tryptophan, and tyrosine as well as cation-π interaction. [36, 50] Sucrose-containing formulation had higher intensity peaks in the tryptophan region (1554cm^{-1}) and amide I region, suggesting better conservation of secondary structure occurred even with increased storage temperature. There was a loss in the β-sheet content with a corresponding gain in the turn and unordered content, which is consistent with the broadening of the amide I band. The results are consistent with FTIR data and show that sucrose protects IgG1 from degradation in the lyophilized state. The cation-π interactions are recognized as an important non-covalent biding interaction relevant to structural biology and likely contribute to protein stability.[40] Also, studies have suggested that cation-π interactions may be involved in dimer formation.[51] The peak ranking of cation-π interaction was as following: C3M, C3MSu, C5M, C7M, C7MSu, and C5MSu. The C5MSu conserved such as interactions by having the least intensity of peak (Figure 15). Conservation of residues involved in these interactions indicated structural stability. These results support the conclusion that the mechanism of protein stabilization by additives during lyophilization depends on the maintenance of the native conformation in the dehydrated state. [24]

Overall, a general correlation was observed between the secondary and tertiary structural data from FTIR, Raman and solid-state fluorescence in the solid state and long term stability of the IgG1 as observed using SE-HPLC and particle analysis (upon reconstitution). However, some exceptions were noted. For example, in this study, C5MSu and C7MSu did not have

stability changes on long-term storage based on SE-HPLC, but the FTIR and Raman data demonstrated minor changes and difference in structure between those two conditions (Figure 5 and 9). Structural results support the conclusion that the structural preservation hypothesis plays a major role on the protein stability in the solid state. However, the lack of perfect correlation between structural change and instability may be considered as a gap. It was observed based on solid-state NMR analysis for the different samples that this gap in correlation between structure and stability on the solid state can be explained by glassy state dynamics (out of the scope for this thesis and therefore data not shown). It was found that NMR relaxation time does correlate with long term stability at the different pH conditions and presence of stabilizer with higher relaxation time corresponds to higher instability. Mannitol in the amorphous state stabilizes the protein along with sucrose as found in literatures. So, glassy state molecular mobility could be a useful tool to explain the gap observed for correlating structural changes to long-term stability.

CONCLUSIONS

The relationship between solid-state protein structure and long-term stability was systematically examined with a series of formulations using a model IgG mAb. It was found that antibody stability in solid state was sensitive to pH. Greater amounts of aggregates were present in low pH formulations and increased with increasing temperature. Also, more secondary and tertiary structure changes in the solid state were observed at low pH. The presence of sucrose in a formulation helped preserve the native structure of the protein in the solid state and inhibited physical instability during long term storage across the pH ranged examined. FTIR analysis of lyophilized antibody formulations also revealed a decrease in the intensity of the native intramolecular β-sheet band in the amide I region in the absence of a lyoprotectant. It appears

that protein should be retained in its native-like state during freeze-drying to assure optimal storage stability in the dried solid.

Both tertiary and secondary structural changes in the solid state generally correlated for most part with long term physical stability in the different formulations at different temperatures and time points. It has been demonstrated that retention of the native conformation (both secondary and tertiary) during lyophilization is an important determinant of stability. Understanding the relationship of physical stability, chemical reactivity, and molecular mobility of the protein is also critical. Based on the major results of the current study it can be concluded that secondary and tertiary structure in the solid state can be good predictor of long term stability, but exceptions were noted, and it likely that glassy state dynamics also can play a role. The results of my study strongly support the "structural preservation" phenomenon enhancing long term stability of protein in the solid state.

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